Original Article

The expression and prognostic value of hypoxia-inducible factor-1α and p53 in non-small cell lung carcinoma

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Background: The study was aimed to explore the expression of hypoxia-inducible factor-1α (HIF-1α) and p53 protein, as well as their correlation with clinicopathological features and survival in non-small cell lung carcinoma (NSCLC) patients.

Methods: The overexpression of HIF-1α and p53 alteration was detected by immunohistochemistry in 50 paraffin-embedded tumor samples from NSCLC patients who received pulmonary surgery and seven benign lung lesion tissue samples. In addition, the correlation between HIF-1α or p53 protein overexpression and clinicopathological features as well as their relationship with epidermal growth factor receptor (EGFR) overexpression was examined by the chi-squared test, while Kaplan-Meier survival analysis was applied to explore their prognostic value.

Results: The results showed that HIF-1α and p53 protein overexpression in NSCLC was remarkably higher than that in benign lung lesion tissue, with a positive rate of 40% and 42%, respectively. HIF-1α overexpression had an unfavorable impact on radiotherapy (P=0.047) and patient survival (P=0.002). However, multivariate analysis showed that HIF-1α was not an independent prognostic factor. There was a significant correlation between the overexpression of HIF-1α and tumor size (P=0.005), nodal metastasis (P=0.014) and pathological stage (P=0.023), while p53 overexpression was only significantly associated with the differentiation level. No correlation was found between p53 overexpression and survival.

Conclusions: Both HIF-1α and p53 protein levels played a crucial role in participating in oncogenesis, and HIF-1α might be a risk factor for survival and a biological marker relevant to radioresistance. However, p53 had no prognostic value for survival.

Keywords: HIF-1α; non-small cell lung carcinoma (NSCLC); p53; prognosis

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Introduction

Lung cancer is the most common malignancy and leading cause of cancer-related death worldwide (1). Non-small cell lung carcinoma (NSCLC) accounts for 75–85% of all lung cancer cases. Despite great advances in diagnosis and therapy, the 5-year survival rate of lung cancer patients remains poor and was reported to be approximately 17% (2). Tumor relapse and metastasis are the main causes of mortality. Several molecular markers have been demonstrated in the literature that are closely related to tumor oncogenesis and progression. Therefore, the detection and analysis of the molecular markers may provide predictive information for the prognosis and may

help to optimize treatment strategies.

Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that mainly responds to hypoxic stress through two transactivation domains: the NH_2-terminal and the COOH-terminal domains (3). It determines the activity of the HIF system and belongs to the basic helix-loop-helix-PAS protein family (4). Activated HIF-1α can help tumor cells adapt to a low oxygen environment by regulating downstream genes that play a crucial role in the basic biological processes of tumor survival and progression. HIF-1α protein has a very short half-life (approximately 5 min) and declines rapidly under normoxia (3). The expression of HIF-1α can be stabilized and accumulated under hypoxia, which occurs in most solid tumors. Under some normoxic conditions, HIF-1α can also be transcribed and synthesized through various signaling factors involving growth factors, cytokines and other signaling molecules (5-7).

HIF-1α expression was reported to be associated with tumor angiogenesis, metastasis, poor prognosis and resistance to therapy in several carcinoma cells (8,9). Neoplasm proliferation and angiogenesis are promoted by elevated HIF-1α expression, whereas the metastasis of cancer cells and angiogenesis are prohibited by down-regulating HIF-1α expression. Overexpression of HIF-1α in colorectal cancer (10), liver cancer (11), and pancreatic cancer (12) patients is more likely to be associated with unfavorable survival. However, the prognostic significance of HIF-1α in NSCLC is inconsistent (13,14). The radiation resistance is partly derived from the presence of hypoxia, and hypoxia-mediated radioresistance was reported to be dependent on the HIF-1α pathway, but the correlation between HIF-1α and radioresistance was only explored in preclinical studies (15-20).

The *p53* tumor suppressor gene is the most commonly identified genetic alteration in human carcinoma. The expression of HIF-1α is down-regulated through binding to wild-type p53 protein (21). Aberration of the *p53* gene is closely associated with oncogenesis. In normal tissue, the *p53* gene plays an important role in maintaining genomic stability by promoting DNA repair, arresting aberrant cells and inducing apoptosis during DNA damage (22). Wild-type p53 protein is a negative regulator of cell proliferation and a positive promoter of apoptosis and often undergoes quick degradation. Mutation of the *p53* gene may prolong the half-life of p53 protein, which usually results in the structural stabilization and accumulation of p53 protein that can be detected by immunohistochemistry (IHC) (23). The inactivation of p53's normal function may be an inducer of increasing malignancy and resistance to chemotherapy (24). The prognostic value of p53 protein overexpression is unclear. Therefore, an intensive understanding of the prognosis of p53 and HIF-1α in NSCLC may result in a more reasonably targeted therapeutic regimen.

The aim of this study was to explore the overexpression of p53 protein and HIF-1α and their prognostic value in NSCLC patients. Additionally, the relationship between HIF-1α and p53 overexpression and resistance to therapy was also analyzed. The correlation between HIF-1α and epidermal growth factor receptor (EGFR) expression is discussed as well.

Methods

Patients and tissue characteristics

Fifty paraffin-embedded NSCLC tumor samples and seven benign lung lesion specimens between September 2001 and February 2004 were randomly selected in this study. Informed consent from all patients and approval from the Medical Ethics Committee of West China Hospital, Sichuan University, were obtained. All patients had received pulmonary lobectomy and mediastinum lymphadenectomy with R0 surgery and did not take any preoperative therapy. There were 32 males and 18 females, aged 43–76 years (mean age: 56 years). All the patient samples were histologically subtyped and graded according to the 3rd WHO (World Health Organization) classification (25) for lung cancer. Among them, there were 21 squamous cell carcinomas, 26 adenocarcinomas, and 3 adenosquamous carcinomas; additionally, 12 were poorly differentiated (G3), and 38 were moderate or well differentiated (G1–G2). Sixteen patients were staged I–II and 34 were staged III-IV based on the 7th edition AJCC (American Joint Committee of Cancer) staging system (26) for lung cancer. Thirty-five cases had intra-thoracic nodal metastasis (N1–3), whereas 15 patients were without lymph node metastasis (N0). All patients underwent 4 cycles of adjuvant platinum-based doublet chemotherapy, and 23 patients staged IIIA with N2 received postoperative elective mediastinal radiotherapy. The benign lung lesions were proven to be inflammatory tissue and were used as controls. Overall survival was calculated from the date of surgical resection to death.

Immunohistochemistry (IHC)

IHC was performed strictly according to the manufacturer's

instructions. The paraffin-embedded tumor samples were cut consecutively into 4-μm-thick sections and were deparaffinized and rehydrated in xylene and ethanol, respectively. Antigen retrieval was accomplished by microwaving the slices treated with sodium citrate buffer. After the slides were washed in distilled water, the tumor specimens were covered with 3% H_2O_2 for 5 minutes to exclude endogenous peroxidase. Non-specific antigen was blocked with normal goat serum for 10 minutes. The slides were exposed to the primary mouse monoclonal anti-EGFR antibody (Chemicon International, Inc.) diluted 1:200 for 30 minutes. The tissue sections were also incubated at 4 ℃ overnight with mouse monoclonal antibody against HIF-1α and p53 (Beijing Zhongshan Biological Company) at a dilution of 1:100. The slides were incubated with a biotinylated secondary anti-mouse antibody for 10 minutes and streptavidin-biotin peroxidase complex solution for 20 minutes. The slices were visualized with diaminobenzidine-tetrahydrochloride (DAB) substrate chromogen solution and then were counterstained with hematoxylin.

Scoring method

HIF-1α-positive cells were identified as exhibiting brown-stained nuclei or brownish-yellow particles, p53-positive cells showed brown granules in the nucleus, and EGFR-positive cells showed brownish-yellow granules in the cytoplasm and cell membrane. Evaluation of the immunohistochemistry results was performed independently by two pathologists who were blinded to all characteristics. The number of stained cells was counted in each field of 200 tumor cells. The positivity ratio comes from the average of 5 random fields under high power (200×). In our immunohistochemistry study, more than 50% nuclear staining of HIF-1α or p53 protein was defined as positive overexpression, while ≥10% EGFR staining was considered positive overexpression.

Statistical analysis

The data were analyzed by SPSS 24.0 version. The chi-squared test was used to examine the association of HIF-1α and p53 overexpression with various clinicopathologic parameters, as well as the relationship between HIF-1α and EGFR. The overall survival of NSCLC patients with HIF-1α or p53 protein overexpression was evaluated using the Kaplan–Meier method and log-rank test. For multivariate analysis of the prognostic factors, the Cox proportional-hazard model was used. P<0.05 was considered statistically significant.

Results

Overexpression of HIF-1α and p53 proteins

The NSCLC tumor showed remarkably different overexpression of HIF-1α and p53 protein from non-neoplastic lung tissue (P=0.045 and P=0.039, respectively) (*Figure 1*). Among them, 40% of tumor samples showed HIF-1α overexpression and 42% of cases displayed p53 protein overexpression (*Tables 1*,*2*). The positive rate of HIF-1α was 53.85% for adenocarcinoma, which was significantly higher than that of 23.81% for squamous carcinoma (P=0.037). The positive rate of p53 protein in adenocarcinoma was 34.60%, which was lower than that in squamous carcinoma (47.60%), but with no significant difference between them (P=0.382).

Correlation between HIF-1α or p53 protein overexpression and overall survival

Patients with negative HIF-1α overexpression had a mean overall survival time of 55 months, which was significantly longer than that of 31 months for HIF-1α overexpression (P=0.002) (*Figure 2*). Cox multivariate analysis of survival showed that HIF-1α overexpression alone was not an independent prognostic factor. Meanwhile, no correlation was found between p53 protein overexpression and overall survival for NSCLC patients in univariate analysis (P=0.131).

Correlation between HIF-1α or p53 overexpression and clinicopathological features

The overexpression of HIF-1α in different subgroups was compared and is summarized in *Table 3*, which shows that the difference in HIF-1α overexpression existed according to tumor size, nodal metastasis and TNM stage subgroup. The HIF-1α overexpression rate in patients with a smaller tumor size (≤3 cm) was significantly lower than that in patients with a larger tumor size (>3 cm) (11.76% *vs.* 54.55%, respectively; P=0.005). HIF-1α overexpression in lymph node metastasis cases was significantly higher than in cases without metastasis (51.43% *vs.* 13.33%, respectively; P=0.014). In addition, with higher TNM stage, the patients were more likely to present with HIF-1α overexpression,

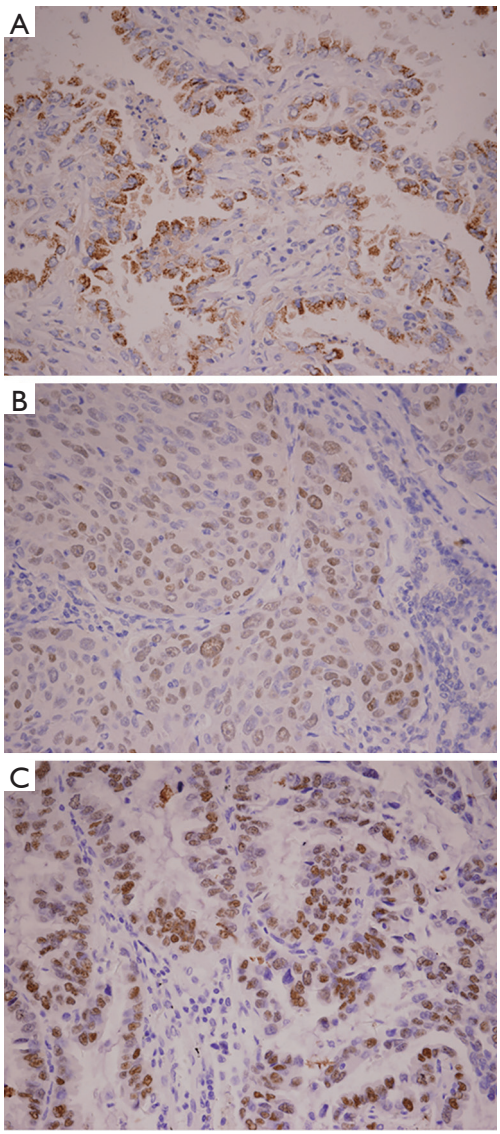

Figure 1 Immunohistochemical staining (×200) for EGFR, HIF-1α and p53 overexpression in lung cancer (EGFR overexpression in A, HIF-1α and p53 overexpression in B and C). EGFR, epidermal growth factor receptor; HIF-1α, hypoxia-inducible factor-1α.

and the rates were 12.50%, 51.72% and 60% for those with stage I–II, III and IV disease, respectively (P=0.023). There was no significant difference in HIF-1α overexpression among other clinicopathological features (such as age, gender and differentiation level). By contrast, only the histological differentiation level showed statistical significance with p53 protein overexpression (P=0.036), with a significantly higher overexpression rate in poorly differentiated tumors. Although p53 protein overexpression was found to be higher in patients aged ≤60 years, male patients, and those with squamous carcinoma histology, poorly differentiated tumors, lymph node metastasis and stage III–IV disease, no statistical significance was detected (P>0.05) (*Table 4*).

HIF-1α overexpression in patients receiving radiotherapy

Twenty-three patients staged as IIIA with N2 had received postoperative adjuvant radiotherapy of the mediastinal lymph nodal region, and the mean survival time for patients with positive HIF-1α overexpression (n=10) was 29 months, which was significantly shorter than that (42 months) for patients (n=13) with negative HIF-1α overexpression (P=0.047) (*Figure 3*).

Correlation of HIF-1α with p53 overexpression and EGFR status

As shown in *Tables 5,6*, there was a significant correlation between EGFR and HIF-1α overexpression (P=0.028). However, no correlation was found between HIF-1α and p53 protein overexpression (P=0.128).

Discussion

HIF-1α is a predominant transcription factor that regulates the transcriptional activity of genes responding to hypoxic

Table 1 HIF-1α overexpression in NSCLC and benign lung lesion tissue

Tissue type	Number of cases	HIF-1α		Positive rate (%)	P value
		Positive	Negative		
NSCLC tissue	50	20	30	40	0.045*
Benign lung lesion	7	0	7	0	–

*, P<0.05. HIF-1α, hypoxia-inducible factor-1α; NSCLC, non-small cell lung carcinoma.

Table 2 p53 protein overexpression in NSCLC and benign lung lesion tissue

Tissue type	Number of cases	p53 protein		Positive rate (%)	P value
		Positive	Negative		
NSCLC tissue	50	21	29	42	0.039*
Benign lung lesion	7	0	7	0	–

*, P<0.05. NSCLC, non-small cell lung carcinoma.

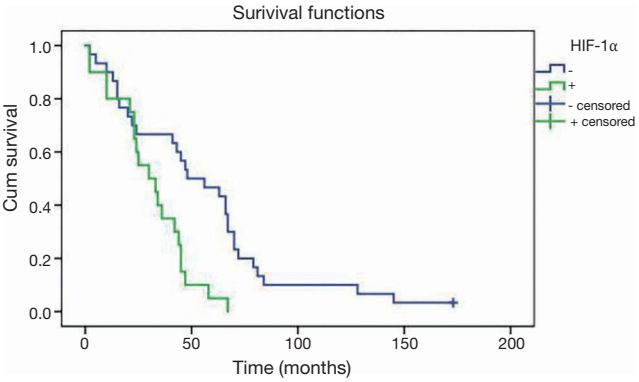

Figure 2 Survival curve with HIF-1α expression (the blue line indicates the survival for patients without HIF-1α overexpression, and the green line indicates the survival for the HIF-1α overexpression subgroup). HIF-1α, hypoxia-inducible factor-1α.

stress. Hydroxylation of HIF-1α proline residues leads to the degradation of HIF-1α protein under normoxic conditions. The activation of HIF-1α can be modulated by several proteins such as pVHL, CBP/P300 and p53 (21). Both HIF-1α and p53 abnormality make contributions to the tumorigenesis of lung epithelial cells. p53 alteration can be detected by IHC and gene sequencing. IHC was adopted in our study because of its reproducibility and high accordance with the genomic state (27,28). It is unclear whether the overexpression of HIF-1α and p53 protein has an adverse influence on the overall survival of NSCLC patients. Our study explored the prognostic value of HIF-1α and p53 overexpression in NSCLC patients by immunohistochemical testing.

HIF-1α and NSCLC

It was previously published that HIF-1α is overexpressed in human carcinomas (29). In our study, the positive rate of HIF-1α overexpression was 40% in NSCLC patients and was significantly higher than that in benign lung lesion tissue (40% *vs.* 0%, respectively; P=0.045), indicating that HIF-1α overexpression might have a strong association with carcinogenesis, tumor progression and metastasis in NSCLC. Previous IHC results showed 32–56% positivity rates of HIF-1α protein overexpression in NSCLC (30-32).

In our study, HIF-1α overexpression in NSCLC was significantly correlated with tumor size (P=0.005), lymph node metastasis (P=0.014) and TNM stage (P=0.023). In the tumor size subgroup analysis, HIF-1α overexpression was mainly distributed in patients with larger tumors. This finding could be explained by the fact that rapid tumor growth aggravated the hypoxic condition while molecular oxygen can diffuse a limited distance. HIF-1α overexpression is more likely to occur in patients with nodal metastasis and tumors of a greater size and higher stage, indicating a close link between HIF-1α and tumor genesis, progression and metastasis. HIF-1α overexpression was independent of the patient's age, sex, and cell differentiation (P>0.05). Similar results were found in several studies (14). Prior studies showed that the positive rate in squamous cell carcinoma is higher than that in adenocarcinoma, but our study showed an opposite result (14). The small size of our study may be one possible interpretation for this discrepancy.

In our study, the Kaplan-Meier and log-rank analysis showed that patients with HIF-1α overexpression had a shorter mean overall survival time of 31 months compared with 55 months for patients without HIF-1α overexpression (P=0.002). Cox multivariate analysis adjusted by other significant variables demonstrated that HIF-1α was not an independent prognostic factor in NSCLC. The result was consistent with the finding from nasopharyngeal carcinoma that HIF-1α is only a risk factor for survival (33). Yang *et al.* recently reported a meta-analysis based on 20 studies published prior to January 2015 indicating that 25% of studies with elevated HIF-1α expression were associated with a poor prognosis in NSCLC patients (14). Therefore, HIF-1α might be a biomarker assisting in the identification of high risk for NSCLC patients. However, an opposite result

Table 3 Correlation between HIF-1α overexpression and clinicopathological features

Clinicopathological features	HIF-1α		Positive rate (%)	P value
	Positive	Negative		
Ages				0.077
≤60	15	15	50.00	
>60	5	15	25.00	
Sex				0.470
Male	14	18	43.75	
Female	6	12	33.33	
Pathologic type				0.109
Squamous carcinoma	5	16	23.81	
Adenocarcinoma	14	12	53.85	
Mixed type	1	2	33.33	
Differentiation level				0.892
G1-2	15	23	53.57	
G3	5	7	41.67	
Tumor size (cm)				0.005*
≤3	2	15	11.76	
>3	18	15	54.55	
Lymph node				0.014*
N0	2	13	13.33	
N1–3	18	17	51.43	
TNM stage				0.023*
I–II	2	14	12.50	
III	15	14	51.72	
IV	3	2	60.00	

*, P<0.05. HIF-1α, hypoxia-inducible factor-1α.

Table 4 Clinicopathologic factors and its relationship with p53 protein overexpression

Clinicopathologic feature	p53 protein		Positive rate (%)	P value
	Positive	Negative		
Ages				0.815
≤60	13	17	43.33	
>60	8	12	40.00	
Sex				0.352
Male	15	17	46.90	
Female	6	12	33.33	
Pathologic type				0.449
Squamous carcinoma	10	11	47.60	
Adenocarcinoma	9	17	34.60	
Mixed type	2	1	66.60	
Differentiation level				0.036*
G1-2	13	26	33.33	
G3 (poorly differentiated)	8	3	72.73	
Tumor size (cm)				0.933
≤3	7	10	41.20	
>3	14	19	42.40	
Lymph node				0.851
N0	6	9	40.00	
N1–3	15	20	42.86	
TNM stage				0.193
I–II	6	10	37.50	
III	11	18	40.00	
IV	4	1	80.00	

*, P<0.05.

was reported in a few studies wherein patients with HIF-1α positive expression had a longer survival than those with HIF-1α negative expression (9). Larger prospective trials are needed to ascertain the prognostic value of HIF-1α.

In patients receiving radiotherapy, the mean survival time for patients with HIF-1α overexpression (n=10) was 29 months, which was significantly shorter than the 42 months in patients (n=13) without HIF-1α overexpression (P=0.047). Thus, HIF-1α overexpression might be a molecular marker that is relevant to radioresistance. Hypoxia is a major influential factor of radioresistance. Up-regulation of HIF-1α often occurs in intratumoral hypoxia. Prior studies have demonstrated that HIF-1α could serve as a potential target to overcome hypoxia-relevant radioresistance. Small-molecule inhibitors or RNA interference (siRNA) targeting HIF-1α could sensitize carcinoma cells in irradiation for many tumor cell lines through cell cycle regulation and the apoptosis-related signaling pathway (15-20). However, the mechanism regarding how HIF-1α influences tumor radiation

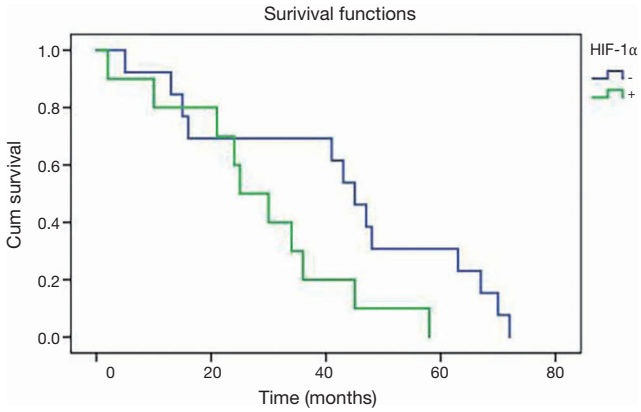

Figure 3 Survival curve based on HIF-1α expression in patients receiving irradiation (the blue line represents the survival for patients without HIF-1α overexpression, and the green line represents the survival for the HIF-1α overexpression subgroup). HIF-1α, hypoxia-inducible factor-1α.

Table 5 Correlation of EGFR and HIF-1α overexpression

EGFR	HIF-1α		Total	P value
	Positive	Negative		
Positive	13	10	23	0.028*
Negative	7	20	27	
Total	20	30		

*, P<0.05. EGFR, epidermal growth factor receptor; HIF-1α, hypoxia-inducible factor-1α.

Table 6 Correlation of p53 and HIF-1α protein overexpression

p53	HIF-1α		Total	P value
	Positive	Negative		
Positive	11	10	21	0.128
Negative	9	20	29	
Total	20	30		

HIF-1α, hypoxia-inducible factor-1α.

sensibility is complicated, and HIF-1α can induce radiation resistance via increasing the transcription of the apoptotic suppressor gene and promoting endothelial cell survival, while HIF-1α can also improve radiation sensitivity by ATP metabolism and proliferation (34). Rigorous clinical research is needed to explore the relationship between HIF-1α and radiation resistance.

Our study reported a significant correlation between EGFR and HIF-1α overexpression (P=0.028). Prior research showed that upregulation of HIF-1α can be mediated by EGFR activation through the PI3K/Akt/mTOR and Mek/Erk pathways (35,36). Park *et al.* reported that the prognostic value of HIF-1α was validated in EGFR negative expression (37), implying that the joint evaluation of biomarkers is more important in the prognosis.

p53 and NSCLC

In our study, the positive rate of p53 overexpression in NSCLC was 42%, a value that is apparently higher than that in benign lesion tissue (42% *vs.* 0%, respectively; P=0.039). Thus, p53 alteration might participate in the oncogenesis of NSCLC. Mitsudomi *et al.* showed that the positivity rate of p53 protein expression was correlated with the antibody used for detection, ranging from 17.5% to 76.8%, and the overall positive rate was 48.2% in NSCLC by IHC, which fits well with our study (23).

In our study, p53 protein overexpression in adenocarcinoma was lower than that in squamous carcinoma (34.6% *vs.* 47.6%, respectively), a result that agrees well with other study results (23,27,28,38). p53 protein overexpression was found to be higher in patients aged ≤60 years and those with squamous carcinoma, poorly differentiated tumors, a tumor size >3 cm, lymph node metastasis, and stage III–IV disease. However, among them, only the histological differentiation level showed statistical significance with p53 protein overexpression, with poorly differentiated tumors showing a significantly higher overexpression rate.

The present study showed no significant association between p53 protein overexpression and overall survival in NSCLC (P=0.131). A meta-analysis covering 22 studies showed that p53 alteration was a poor prognosis marker only in adenocarcinoma of NSCLC (23). In addition, similar results were found in several subsequent studies (39,40). In our study, no prognostic value of p53 overexpression was found in either squamous carcinoma or adenocarcinoma. However, the prognostic value of p53 expression in NSCLC has been inconsistent among different studies (23,27,28,39-41). The possible explanation might be that tumorigenesis and cancer development are complicated and influenced by various factors.

In 27 patients only receiving chemotherapy, we observed a mean overall survival of 47 months in patients (n=9) with p53 protein overexpression, which was shorter than 57 months for patients (n=18) without overexpression

(P=0.443). Shiga *et al.* reported a similar result in head and neck cancer (42). *In vitro* studies have also identified drug resistance in p53-mutated lung cancer cells (24). Tung *et al.* proved the hypothesis that mutant p53 might induce cisplatin resistance via upregulating Nrf2 expression (43), implying that NSCLC patients with normal p53 function may benefit more from adjuvant chemotherapy.

In our study, no statistical correlation was found between p53 and HIF-1α protein overexpression. Both HIF-1α and tumor suppressor p53 mediate the cellular response to hypoxia stress. Loss of the p53 gene is correlated with upregulation of HIF-1α (21). p53 mutation could induce the resistance of p53-mediated apoptosis under hypoxia. In addition, HIF-1α could inhibit tumor cell apoptosis caused by irradiation via suppressing the expression of p53 (44). However, how the two factors interact to decide cell fate remains unclear.

In conclusion, our study illustrated that HIF-1α and p53 proteins are both overexpressed in NSCLC. HIF-1α might be a risk factor for overall survival and a biological marker relevant to radiation resistance. There was a significant correlation between HIF-1α and EGFR expression. Although our study failed to endorse the role of p53 abnormality as an unfavorable outcome in NSCLC, it clearly supports its critical role in the development of lung carcinoma. The prognostic value of HIF-1α and p53 expression needs further study.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: Informed consent from all patients and the approval from Medical Ethics Committee of West China Hospital, Sichuan University has been obtained, and the ID is 2018(50).

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